

Navy Case No. 79,212

PATENT APP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

## APPLICATION FOR LETTERS PATENT

TO ALL WHOM IT MAY CONCERN:

BE IT KNOWN THAT Alok Singh, Mehran Pazirandeh, Paul E. Schoen, Michael A. Markowitz, and J. Matthew Mauro, who are citizens of the United States of America, and are residents of Springfield, Virginia, Silver Spring, Maryland, Alexandria, Virginia, Burke, Virginia, and Silver Spring, Maryland, respectively, have invented certain new and useful improvements in "PASSIVATION OF NERVE AGENTS BY SURFACE MODIFIED ENZYMES STABILIZED BY NON-COVALENT IMMOBILIZATION ON ROBUST, STABLE PARTICLES" of which the following is a specification:

Please Contact Preparer:  
Amy Loch Rassing  
Reg. No. 45,814  
Tel: 202-404-1556  
Date: October 31, 2000

PASSIVATION OF NERVE AGENTS BY SURFACE MODIFIED ENZYMES  
STABILIZED BY NON-COVALENT IMMOBILIZATION ON ROBUST, STABLE  
PARTICLES

**Field of the Invention**

The present invention relates to a detoxification/decontamination system for nerve agents which has long term stability over a wide temperature range.

**Background of the Invention**

Nerve agents pose a growing threat to society whether they are released accidentally or deliberately. Current means to counter threats from nerve agents, although temporarily effective, are not adequate. Currently, activated charcoal is used to filter nerve agents from air and water; bleach solution or jet fuel is used for decontaminating protective gear.

However, these methods use compositions which have undesirable properties including corrosiveness, flammability, and toxicity. Moreover, these methods can only be used on a small scale, and they are not effective over an extended period of time.

Delivery of active enzyme systems to counter and detoxify chemical and biological warfare agents is a promising and active area of research. While some enzymes in their native form have exhibited effectiveness against nerve agents, there are still many challenges

1 in developing effective detoxification systems, including preservation of high catalytic  
2 activity in real conditions, stability of the enzyme system after prolonged storage, suitable  
3 means of delivery, and accessibility of enzymes to threat agents.

4 "Detoxifying Nerve Agents", C&E News September 15, 1997, page 26 reports the  
5 current state of the art for detoxification of nerve agents, with special reference to efforts on  
6 the part of the U.S. Army. A class of enzymes that is known to catalyze the hydrolysis of  
7 organophosphate compounds has been investigated for potential decontamination. The  
8 organophosphate anhydrolases (OPAA: EC3.1.8.2) catalyze the hydrolysis of many G-type  
9 chemical warfare nerve agents. Specifically, these enzymes have activity against compounds  
10 such as sarin, soman, and GF (O-cyclohexyl methylphosphono fluoridate). Covalently  
11 linking enzymes to solid substrates and embedding enzymes in polymer matrices are the two  
12 most common means for enzyme immobilization. However, the covalent chemistry required  
13 for linking an enzyme to a substrate often adversely affects the enzyme's activity. Enzymes  
14 embedded in polymer matrices are not accessible freely to the agents present in the  
15 surrounding medium.

16 Branner-Jorgensen, in U.S. Patent No. 4,266,029, disclose immobilizing enzymes on a  
17 mineral oxide which has been coated with gelatin and glutaraldehyde. However, these  
18 enzymes are used in fluidized bed operations, and there is no indication that these enzymes  
19 can be used to detoxify nerve agents.

20 Doctor et al., in U.S. Patent No. 5,366,881, disclose mutant cholinesterase which can  
21 be used for detoxifying organophosphates. However, to maintain the activity of the  
22 cholinesterases, oximes are added.

Recently, LeJeune and coworkers reported immobilizing phosphotriesterases onto polyurethane polymers for decontamination purposes (LeJeune et al., *Biotechnology and Bioengineering* 54:105-114, 1997). However, there are several drawbacks to using polyurethane for immobilizing phosphotriesterases. In addition to being an environmentally unfriendly polymer, polyurethane may not afford the maximal protein stability that can be achieved in the protein's native environment. In addition, the enzymes used in these studies have not been selected for use under field conditions, and suffer many drawbacks, including inhibition by substrate, low turnover, and low stability. Watkins et al., *Biological Chemistry* 272:25596-25601, 1997) have demonstrated enhanced rate of hydrolysis of phosphorus-fluorine bonds by phosphotriesterases using engineered enzymes.

## Summary of the Invention

It is another object of the present invention to provide a mutagenesis and selection/screening method to obtain enzymes with the desired catalytic and stability properties.

It is a further object of the present invention to modify the enzymes obtained for

1 non-covalent immobilization on the surface of polymerized vesicles.

2 It is another object of the present invention to modify the enzymes obtained for  
3 non-covalent immobilization on the surface of silica particles.

4 The following method is used to produce effective agents for  
5 detoxifying/decommissioning nerve agents:

- 6 (1) Select a suitable enzyme.
- 7 (2) Modify the enzyme by incorporating anchor sites for  
8 linking it to a target surface without destroying the catalytic activity of the enzyme.
- 9 (3) Construct a stable carrier to accommodate and bind  
10 the selected enzyme.
- 11 (4) Non-covalently link the enzyme to the colloids of  
12 surface metal iminodiacetate groups and/or nitrilotriacetic acid groups.

13 Once an enzyme has been selected for its catalytic and stability properties, the enzyme  
14 is further modified for non-covalent immobilization on the surface of polymerized vesicles.  
15 The immobilization technique is the subject of U.S. Patent No. 5,663,387, the entire contents  
16 of which are hereby incorporated by reference. Polymerized liposomes are a prime substrate  
17 for immobilizing active enzymes because they retain their structural integrity in adverse  
18 chemical and physical environments, provide a native environment for enzymes to sustain  
19 their activity, and provide higher surface area to facilitate easy access of medium to enzymes.

20 Silica particles can also be used as substrates for non-covalent enzyme  
21 immobilization, because these particles have high surface area and retain their structural  
22 integrity in adverse chemical and physical environments. Silica particles with surface IDA

groups can be formed in at least one of two ways:

*Sub a1* (1) Silica particle precursors, such as TEOS or TMOS are to be co-hydrolyzed with IDA-modified alkoxysilanes using the Stober process (Stober et al., Journal of Colloid Interface Science 26: 62, 1968); or

(2) IDA alkoxysilanes are grafted to the surface of silica particles using well established procedures, Bradley et al., Langmuir 6: 792, 1990; Van Blaaderen et al., Langmuir 8:2921, 1992). After forming a metal IDA salt or a metal NTA salt, non-covalent enzyme immobilization can proceed as previously described. Since enzymes immobilized onto silica or other inorganic particles can be packed into a variety of chromatographic columns (liquid HPLC), they readily lend themselves to simultaneous continuous-flow catalytic processing of multiple toxic agents. This appears to be the first time that surface modified enzymes have been immobilized on silica particles.

### Detailed Description of the Invention

According to the present invention, using either a liposome or silica particle, customized immobilization protocols can be developed and optimized for storing the enzyme systems under otherwise adverse conditions.

*Sub a2* Examples of enzymes which are useful in detoxifying nerve agents are thioesterases, although the process of the present invention can be used with any type of enzyme useful for destroying waste materials. One example of this is lipases, which are used for digesting waste onboard ships. The enzymes are genetically engineered to include a poly-His tail as

1 well as other stabilizing amino acid substitutions. Non-covalent enzyme immobilization on  
2 polymerized liposomes was effected by co-polymerizing amphiphiles containing metal salts  
3 of iminodiacetic acid or nitrilotriacetic acid with other polymerizable amphiphiles and then  
4 binding the enzyme to the iminodiacetic acid-metal salts or NTA-metal salts on the outer  
5 surfaces of the vesicles. This technique relies on the strong binding affinity between  
6 iminodiacetate salts or NTA salts and polyhistidine, which has been made available on the  
7 surface of the enzyme selected for immobilization through genetic engineering. The  
8 enzymes that can be used for this technique are those enzymes that have appropriately  
9 reactive surface available histidines or which have a histidine tag that can be added through  
10 site specific metagenesis. This includes, of course, polyhistidine. Histidine forms a strong  
11 bond with iminodiacetate salts, such as copper, zinc, cobalt, and nickel iminodiacetate salts,  
12 and nitrilotriacetic acid salts, such as copper, zinc, cobalt, and nickel salts. The main  
13 criterion for this process to be effective is that the binding site on the enzyme be far away  
14 from or innocuous to the function of the enzyme's catalytic site. While silica is the  
15 preferred inorganic surface because it is relatively inexpensive and its properties are well  
16 understood, any type of metal oxide ceramic particles that can be formed similar to the Stober  
17 process starting with a metal alkoxide precursor can be used. Other types of inorganic  
18 surfaces that can be used in the process of the present invention include alumina, baria,  
19 titania, and gircinia.

20 *Bachma3* Bachmair et al., in U.S. Patent 5,646,017; 5,496,721; 5,196,321; 5,132,213; and  
21 5,093,242, the entire contents of which are hereby incorporated by reference, disclose  
22 methods for designing or modifying protein structure at the protein or genetic level to

1 produce proteins having specified amino-termini in vivo or in vitro. These methods can be  
2 used to produce proteins having amino-termini on enzymes wherein genes encoding the  
3 enzymes can be made to encode an amino acid of the desired class at the amino-terminus so  
4 that the expressed enzyme exhibits a predetermined amino-terminal structure which renders is  
5 metabolically stable and able to bind to metal salts of iminodiacetic acid which are  
6 copolymerized with amphiphiles. Preferably, the amino-terminal structure is histidine,  
7 although C-terminal or internal polyhis sequences will usually be satisfactory as well.

8 A DNA sequence containing nucleotides coding for the enzyme of interest, as well as  
9 nucleotides which code for an amino acid sequence at the N-terminus or C-terminus of the  
10 enzyme such as histidine which strongly bind to metal iminodiacetate or nitrilotriacetic acid  
11 salts are operably linked to a promoter that will permit expression of the enzyme in the cells  
12 of interest for production thereon. This enzyme cassette is introduced into cells for production  
13 of the stabilized enzyme, after which the stabilized enzymes are recovered therefrom by  
14 conventional means.

15 *but at* The enzymes useful in detoxifying nerve agents are attached to iminodiacetate salt  
16 groups on the surface of silica particles formed by co-hydrolyzing TMOS with an  
17 IDA-alkoxysilane derivative. The IDA-alkoxysilane accounted for 5 weight percent of the  
18 total silica content. After particles were synthesized using the Stober procedure, the copper  
19 salt of the surface IDA groups was formed by adding an aliquot of 20% aqueous  $\text{CuSO}_4$   
20 solution (wt/wt) to the dry particles, and then suspending the particles using mild sonication  
21 or vortex mixing. The suspension was centrifuged and the supernatant was removed. This  
22 procedure was repeated, and the resulting blue silica particles were washed with water by



1 adding the water to the particles, suspending the particles in solution, and then centrifuging  
2 the suspension and removing the supernatant. This procedure was repeated three times.  
3 Then, an aliquot of the thioesterase in 0.05 M phosphate buffer, pH 7.2., was added to a  
4 suspension of the particles in the same buffer. The suspension was incubated at 4°C for three  
5 hours. The particles were then centrifuged and the supernatant was removed. The particles  
6 were then washed using the phosphate buffer described above. All operations involving the  
7 enzyme were performed at 4°C. After the final washing, the particles were resuspended in  
8 the buffer and stored for future use. The activity of the immobilized enzyme was confirmed  
9 using standard procedures.

### Examples

#### 1. Cloning and Modifying Enzyme

12 The gene for thioesterase-1 (TE-1) of E. coli strain JM109 was cloned using a  
13 modification of the procedure published in Escherichia coli: thioesterase I. Molecular  
14 cloning and sequencing the structural gene and identification of a periplastic enzyme, Hyeson  
15 Cho, John L. Carona (1993) Journal of Biological Chemistry 26:9238-9245.

16 Briefly, amplified DNA encoding the TE-1 protein and appropriate flanking nucleotide  
17 sequences was ligated into the DNA vector PCR 2.1 (Invitrogen). After preparing of 140  
18 micrograms of the PCR2.1-TE1 vector DNA from 100 ml overnight culture, the engineered  
19 TE-1 fragment was liberated from the intermediate vector by digestion of 10 micrograms of  
20 this DNA with 20 units each of the restriction endonucleases NdeI and XhoI at 37°C  
21 overnight. The liberated TE-1 coding fragment was purified electrophoretically on a 2%  
22

1 agarose gel. The stained gene fragment was excised from the gel and subsequently obtained  
2 free of agarose using commercial products (Qiagen).

3 *Sulab* The gene for N-terminal polyhistidine-modified TE-1 was prepared by enzymatically  
4 ligating approximately 300 mg of the gene fragment described above with about 100 ng of  
5 pProEx-1 vector DNA (Life Technologies) previously digested with NdeI and XhoI enzymes  
6 and dephosphorylated with calf intestinal alkaline phosphatase. Transformed E. coli  
7 DH5 $\alpha$ F'LacI<sup>r</sup> cells (Life Technologies) were screened for the presence of the TE-1 inserted  
8 gene by electrophoretic analysis of differential whole-cell protein profiles of cells taken from  
9 small scale cultures grown plus and minus 1 mM isopropylthiogalactopyranoside (IPTG)  
10 chemical inducer.

11 TE-1 was purified from 100 ml cell culture (LB/50 micrograms/ml carbenicillin)  
12 induced at 30°C with 1 mM for about two hours (OD<sub>600</sub> at induction ~0.6). Cell  
13 resuspension, sonic lysis, and chromatographic purification were carried out according to  
14 published procedures published in Protein Biotechnology (1993) Felix Franks, Human Press,  
15 Totwa, NJ, and references cited therein. The final eluted TE-1 product, 14 ml, was dialyzed  
16 for three days against 3 L 50 mM potassium phosphate buffer, pH 7.2. The dialyzed product  
17 was concentrated in two stages to 0.65 ml using Centriprep-10 and Centricon-10 centrifugal  
18 concentrators at 4°C. The final protein concentration of 0.35 mg/ml was evaluated against  
19 bovine serum albumin standard protein using a Bio-Rad (Bradford method) assay kit.

20  
21 2. Assay of Enzymatic Activity Immobilized TE-1

22 Samples of TE-1 immobilized on IDA silica were assayed for their ability to

1 hydrolyze p-nitrophenyl propionate (SIGMA) according to published procedures. In a typical  
2 assay, equivalent amounts of silica/enzyme slurry, or appropriate control samples, in 10 to 20  
3 microliters were added to a 1.5 ml polypropylene conical microcentrifuge tube that contained  
4 0.97 ml physiologically buffered saline (PBS) at pH 7.2, 3%v/v acetone, and 0.370 mM  
5 p-nitrophenyl propionate. Each tube was capped, oriented on its side, and shaken at 225  
6 RPM at 30 C for 30 minutes. After 30 minutes, each sample was immediately centrifuged at  
7 room temperature for exactly one minute. Then, 0.90 ml of each sample was removed and  
8 immediately assayed spectrophotometrically at 346 nm. In one such assay, the background  
9 corrected results were as follows:

<u>Sample</u>	<u>Activity (OD<sub>346</sub>units/min x 10<sup>3</sup>)</u>
Cu <sup>2+</sup> + IDA silica + TE-1	5.12
Cu <sup>2+</sup> + IDA silica	0.47
IDA silica	0.67

### 3. Formulation and Catalytic Activity of Cu<sup>2+</sup>-IDA Silica Particles

The silica particles were formed by co-hydrolyzing TMOS with an IDA-alkoxysilane. The IDA-alkoxysilane accounted for 5 weight% of the total silica content. After particle synthesis using the Stober procedure, the copper salt of the surface IDA groups was formed by adding an aliquot of aqueous 20% CuSO<sub>4</sub> solution, w/w, to the dry particles, and then suspending the particles using mild sonication and vortex mixing. The suspension was centrifuged and the supernatant was removed. This procedure was repeated, and then the resulting blue silica particles were washed with water by adding the water to the particles,

1 suspending the particles in solution, and then centrifuging the suspensions and removing the  
2 supernatant. This procedure was repeated three times. A small portion of these particles was  
3 further washed with an aqueous saturated EDTA tetrasodium salt solution in a similar  
4 manner. Upon adding the EDTA solution, the supernatant turned from clear to blue and the  
5 particles turned from blue to white, demonstrating that copper ions had been bound to the  
6 IDA groups on the surface of the particles.

7 Then, the  $\text{Cu}^{2+}$ -IDA particles were suspended by mild sonication and vortex mixing in  
8 1 ml of 0.005 M aqueous phosphate buffer, pH 7.2. Then, 40 $\mu\text{L}$  of this suspension was added  
9 to a test tube. 160 $\mu\text{L}$  of the buffer was added, and the resulting suspension was cooled to  
10 4°C. After three hours at 4°C, the catalytic activity of the particles was tested using a  
11 thioesterase assay. The particles exhibited catalytic activity as follows:

12  $\text{Cu}^{2+}$  +IDA silica particles, 0.47  $\text{OD}_{346}$  units/min  $\times 10^3$

13 IDA silica particles, 0.67  $\text{OD}_{346}$  units/min  $\times 10^3$

14 This example demonstrates that the  $\text{Cu}^{2+}$  +IDA particles have no catalytic activity in  
15 the absence of bound thioesterase.

#### 16 17 4. Binding and catalytic Activity of Thioesterase on $\text{Cu}^{2+}$ -IDA Silica Particles

18 Polyhistidine tagged thioesterase was noncovalently attached to copper-IDA groups  
19 on the surface of silica particles made as in Example 1 in the following manner: 40 $\mu\text{L}$  of the  
20 suspension of the  $\text{Cu}^{2+}$  +IDA silica particles in 1 ml of 0.05M aqueous phosphate buffer, pH  
21 7.2., suspension was added to a test tube. 160 $\mu\text{L}$  of the buffer was added, and the resulting  
22 suspension was cooled to 4°C. Then, 10 $\mu\text{L}$  of the thioesterase in the phosphate buffer was

1 added to this suspension, which was then incubated at 4°C for three hours. The particles  
2 were centrifuged and the supernatant was removed, making sure that the silica did not go dry.  
3 The particles were washed using the phosphate buffer as described above. Eight mL of the  
4 buffer was added to the particles, which were then suspended with mild sonication,  
5 centrifuged, and the supernatant removed. This washing procedure was repeated six times.  
6 All operations involving the enzyme were performed at 4°C. After the final washing, the  
7 particles were resuspended in 1 mL of the buffer and stored for future use. The activity of the  
8 immobilized enzyme was confirmed using standard procedures. This sample, Cu<sup>2+</sup> +IDA  
9 silica + TE-1 showed an activity of 5.12 OD<sub>346</sub> units/min x 10<sup>3</sup>. This example demonstrates  
10 the sustained activity of polyhistidine modified thioesterase bound to the Cu<sup>2+</sup> -IDA groups  
11 on the silica particles.

##### 12 5. Binding and Catalytic Activity of thioesterase on IDA with Silica Particles

13 The Cu<sup>2+</sup> -IDA silica particles that had been washed with saturated aqueous  
14 tetrasodium EDTA solution were resuspended in 1 mL of 0.05 M aqueous phosphate buffer at  
15 pH 7.2. 40μL of the suspension of this suspension was added to a test tube. 160μL of the  
16 phosphate buffer was added, and the resulting suspension was cooled to 4°C. Then, 10μL of  
17 thioesterase in phosphate buffer was added to this suspension, which was then incubated at  
18 4°C for three hours. The particles were centrifuged and the supernatant was removed,  
19 making sure that the silica did not go dry. The particles were washed using the phosphate  
20 buffer as described above. Eight mL of the buffer was added to the particles, which were  
21 then suspended with mild sonication, centrifuged, and the supernatant removed. This  
22

1 washing procedure was repeated six times. All operations involving the enzyme were  
2 performed at 4°C. After the final washing, the particles were resuspended in 1 mL of the  
3 buffer and stored for future use. The catalytic activity of these particles, as determined by the  
4 thioesterase assay, was significantly less than the activity of the enzyme bound to the Cu<sup>2+</sup>  
5 -IDA particles. This example demonstrates that binding of the enzyme to the Cu<sup>2+</sup> -IDA  
6 groups on the silica particles is required for optimal catalytic activity.

7 The method of the present invention provides means for stabilizing enzymes in such a  
8 fashion that the enzymes, by virtue of their non-covalent bonding to the liposomes or silica,  
9 are readily available to act on their substrates. The present invention provides an effective  
10 system that uses the efficiency and selectivity of enzymes in catalysis and utility of surfaces to  
11 provide stability to sophisticated enzyme architecture.

12 The foregoing description of the specific embodiments will so fully reveal the general  
13 nature of the invention that others can, by applying current knowledge, readily modify and/or  
14 adapt for various applications such specific embodiments without departing from the generic  
15 concept, and, therefore, such adaptations and modifications should and are intended to be  
16 comprehended within the meaning and range of equivalents of the disclosed embodiments. It  
17 is to be understood that the phraseology or terminology employed herein is for the purpose of  
18 description and not of limitation.